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# Uptake of fluorescent nano beads into BY2-cells involves clathrin-dependent and clathrin-independent endocytosis

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ABSTRACT

**To follow endocytosis in BY-2 cells we made use of fluorescent nano beads. Beads with 20 nm in diameter were internalised rapidly and accumulated partially in compartments also labelled by the endocytic marker FM4-64. Studies in BY-2 cells and protoplasts revealed that larger beads (100 nm) were excluded from uptake into turgescent and plasmolysed cells while protoplasts were able to internalise beads with a diameter of up to 1000 nm. Endocytosis of beads was only partially inhibited by the clathrin-specific inhibitor Ikarugamycin and strongly blocked by wortmannin. These results imply that uptake of beads involves clathrin-dependent and clathrin-independent endocytic mechanisms and supports the hypothesis that clathrin-independent endocytosis plays a general role in plants.**



## 1. Introduction

Over the past years understanding of the molecular processes which underlay endocytosis in plants has increased rapidly. However, the main focus has so far been on clathrin-dependent endocytosis and there is only limited evidence for clathrin-independent endocytosis in plants [1–3]. In our previous study on BY-2 cells we demonstrated that internalisation of glucose does not only occur via transporters in the plasma membrane but also involves clathrin-independent endocytosis [4]. We also speculated that clathrin-independent endocytosis may be a common mechanism for the endocytic uptake of nutrients.

In animal cells clathrin-independent pathways were found to be involved in numerous cellular processes such as polarisation, motility, regulation of signalling and normal cell growth [5]. It thus seems likely that clathrin-independent endocytosis does also play an important role in plants and may be involved in many more cellular processes than currently assumed.

To investigate clathrin-independent pathways the generally used endocytic membrane marker FM4-64 cannot be employed because endocytosis of FM4-64 has been shown to strongly depend on clathrin [6]. We therefore used externally applied fluorescent nano beads of different sizes to follow endocytosis in BY-2 cells and protoplasts. The nano beads are believed to be included into endocytic vesicles and thus function as a marker of vesicular cargo which in turn allows to follow the endocytic pathway in cells. The uptake of nano beads is limited by the dimension of the endocytic vesicles forming at the plasma membrane. One can thus also gain information about the diameter of endocytic vesicles by applying nano beads of different sizes. Incubation of BY-2 cells and protoplasts in small nano beads (20 and 40 nm) revealed a fast internalisation of beads. Uptake of larger beads (up to 1000 nm) was only found in protoplasts. Endocytosis of nano beads could partially be blocked by addition of Ikarugamycin (IKA), an inhibitor of clathrin-dependent endocytosis. Endocytosis of nano beads was also inhibited by the addition of wortmannin. Treatment of cells with wortmannin led to the accumulation of beads in wortmannin induced compartments implying that endocytosis of beads depends on PI3-kinases.

Together the results demonstrate that small nano beads provide a useful tool to follow endocytosis in plant cells. Inhibitor studies imply that uptake of nano beads involves both, clathrin-dependent and clathrin-independent endocytosis. This points to a role of at least two endocytic mechanisms in the constitutive uptake of membrane and fluid in plant cells.

*Abbreviation:* IKA, Ikarugamycin

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## 2. Materials and methods

### 2.1. Cell culture and protoplast isolation

BY-2 cells were cultured in Linsmaier and Skoog medium (Duchefa Haarlem, Netherlands) supplemented with sucrose (30 g/l), 2,4-dichlorophenoxyacetic acid (1 mg/ml), and thiamine (1 mg/ml). Subculturing was done twice a week by adding 10 ml of the cell suspension into 100 ml of new medium. Cultures were maintained in the dark, at 25 °C, under rapid shaking (100 rpm).

Protoplasts were isolated as described before [4] and stored in wash solution (30 mM $CaCl_2$, 30 mM KCl, 20 mM MES, pH 6.5 adjusted to 430 mosmol/kg with sorbitol) at 4 °C until use.

### 2.2. Drug treatment

For drug treatment experiments, aliquots of wortmannin (Roth Karlsruhe, Germany) and Ikarugamycin (IKA) (Biomol Hamburg, Germany) solution (stock at 1 mM in DMSO) were added to tobacco BY-2 cells to a final concentration of 30 and 10 μM, respectively. BY-2 cells were incubated in IKA or wortmannin for 30 min before use for microscopic analysis.

### 2.3. Confocal imaging

BY-2 cells and protoplasts were investigated with a Leica TCS SP5 II spectral confocal microscope (Leica Microsystems). Images were acquired with an HCX PL APO CS 40.0 × 1.30 OIL UV objective. Nano beads (F-8888 and F-8795 Invitrogen, Darmstadt, Germany) were used as a 1:1000 v/v dilution in either BY-2 culture media or wash solution. Directly before microscopic investigation nano bead solutions were sonicated for 10 min to avoid agglomeration. Nano beads were excited with the 488 nm line of a 25 mW argon laser and fluorescence was detected at 505–580 nm. For plasma membrane staining and monitoring of endocytosis BY-2 cells and protoplasts were incubated in 10 μM FM4-64 (Invitrogen Darmstadt, Germany) for 10 min. FM 4-64 was excited with the 488 nm line of a 25 mW argon laser and fluorescence was detected at 630-700 nm. Images were analysed with IMAGEJ software (National Institutes of Health) and Leica Confocal Software (Leica Microsystems CMS GmbH\sLeica LAS AF Lite). For analysis of the relative intracellular fluorescence of FM4-64 the area just below the outline of the protoplasts (marked by FM4-64) was taken as a measure of the intracellular fluorescence intensity. The relative intracellular fluorescence is given as the ratio of the intracellular fluorescence to the whole cell fluorescence. To quantify the internalisation of nano beads the fluorescent intensity of nano beads inside the cell was calculated and divided by the cell area. The fluorescent intensity per area is given as a change in fluorescence relative to the start of observation. Values are presented mean ± SEM.

## 3. Results and discussion

### 3.1. Fluorescent nano beads can be used to follow endocytosis in BY-2 cells

To follow endocytosis in BY-2 cells we made use of fluorescent nano beads (also called microspheres). These beads are spherical particles which come in different sizes ranging from 20 nm to more than 1 μm. They are formed from polystyrene and are loaded with dyes to create intensely fluorescent beads that show little photobleaching. In the context of research on endocytosis they have so far mainly been applied to study phagocytosis in mammalian cells (see for example [7]). Nano beads have also been applied to follow fluid phase endocytosis in sycamore cultured cells [8]. However, in these cells uptake of nano beads was hindered by the severe clumping of beads and by their tenacious adherence to the cell walls. Uptake of beads was only visible in electron micrographs of protoplast incubated with beads for 12 h [8]. To test the suitability of fluorescent beads to follow endocytosis in BY-2 cells, nano beads of 20 nm in diameter were added to the cells. In all cells we found a fast up-

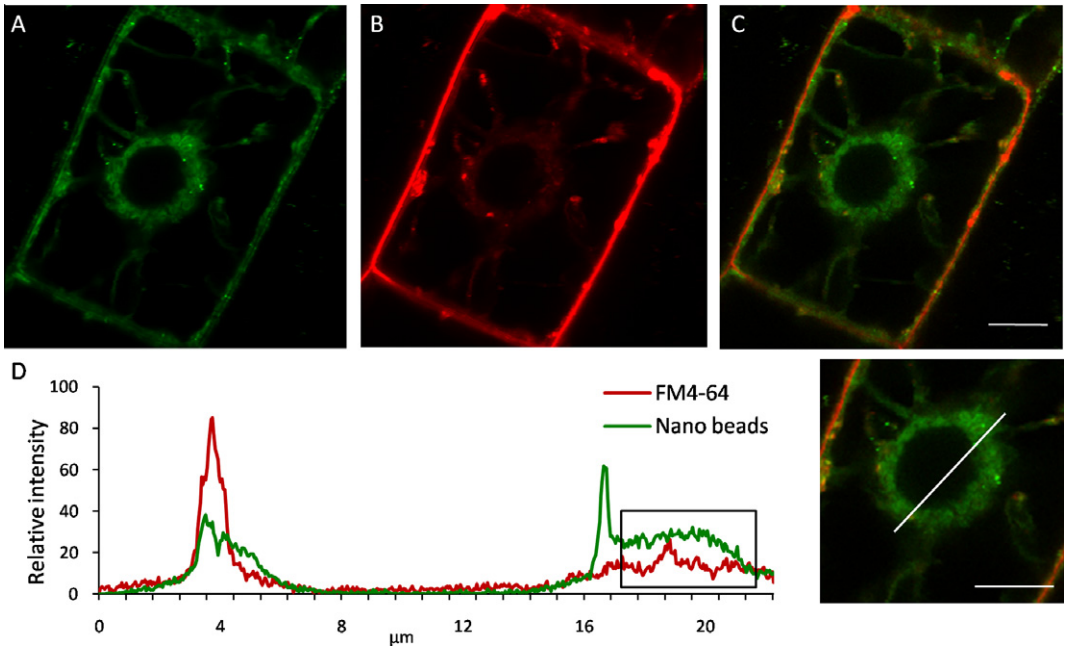


**Fig. 1.** Endocytic uptake of 20 nm nano beads into BY-2 cells. (A–C) Fluorescent images of BY-2 cells 15 min after addition of 20 nm nano beads and FM4-64. Nano beads accumulate in bright spots in the cytosol (A) which partly colocalise with endocytic compartments labelled by FM4-64 (B, C). (D) Intensity profile of nano beads (green) and FM4-64 (red) taken from the white line indicated in the close up. Peaks most likely represent accumulation of fluorescence in endosomes. Fluorescent signals highlighted by the box results from a diffuse signal in the cytosol corresponding to small vesicles below the resolution limit. Scale bars (A–C) = 10 μm, (D) = 10 μm.

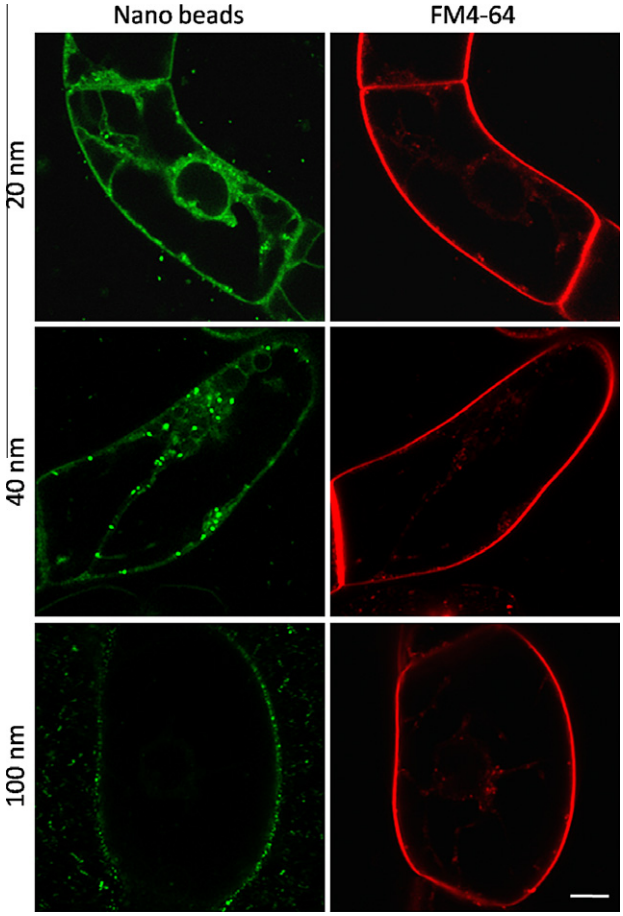


**Fig. 2.** Nano beads of 100 nm and above are excluded from internalisation into BY-2 cells. Fluorescent images of BY-2 cells incubated with nano beads of different sizes (as indicated). 20 and 40 nm beads are visible in bright spots in the cytosol. One hundred nanometer beads adhere to the cell wall but are excluded from the endocytic internalisation. FM4-64 labels the endomembrane system, indicating that endocytosis occurred normally. Scale bars = 10 μm.

take of nano beads leading to a bright intracellular fluorescence. A representative example of a BY-2 cell 15 min after addition of the beads is shown in Fig. 1A–C. The fluorescent signal was visible in bright spots and as a diffuse distribution in the cytosol. The bright spots were highly mobile and moving along cytosolic strands (Supplemental data, Movie 1). During the time of observation (max. 1 h) we did not find any fluorescence of nano beads in the vacuole (not shown). In some experiments we also observed adherence of nano beads to the cell wall but even in these cells we could clearly resolve internalised nano beads. To confirm that internalisation of nano beads reflects endcytic uptake of the beads we compared the fluorescent distribution of nano beads with the localization of the endocytic marker FM4-64. As shown in Fig. 1B FM4-64 was also found in the cytosol and in some spots of different size. Some but not all of the bright spots are marked by both, fluorescent nano beads and FM4-64 (Fig. 1C). This partial colocalisation is confirmed by a fluorescent intensity profile (Fig. 1D). Peaks in the intensity profile most likely represent accumulation of fluorescence in endosomes. The first peak is found in the fluorescent signal of both, nano beads and FM4-64 while the second peak lacks a large FM4-64 signal (Fig. 1D). This shows that nano beads do not only accumulate in endosomes stained by
FM4-64 but also use different endocytic pathways leading to accumulation in different endosomes. The intensity profile also includes signals from the diffuse distribution in the cytosol (highlighted by the box) which is found in the FM4-64 and in the

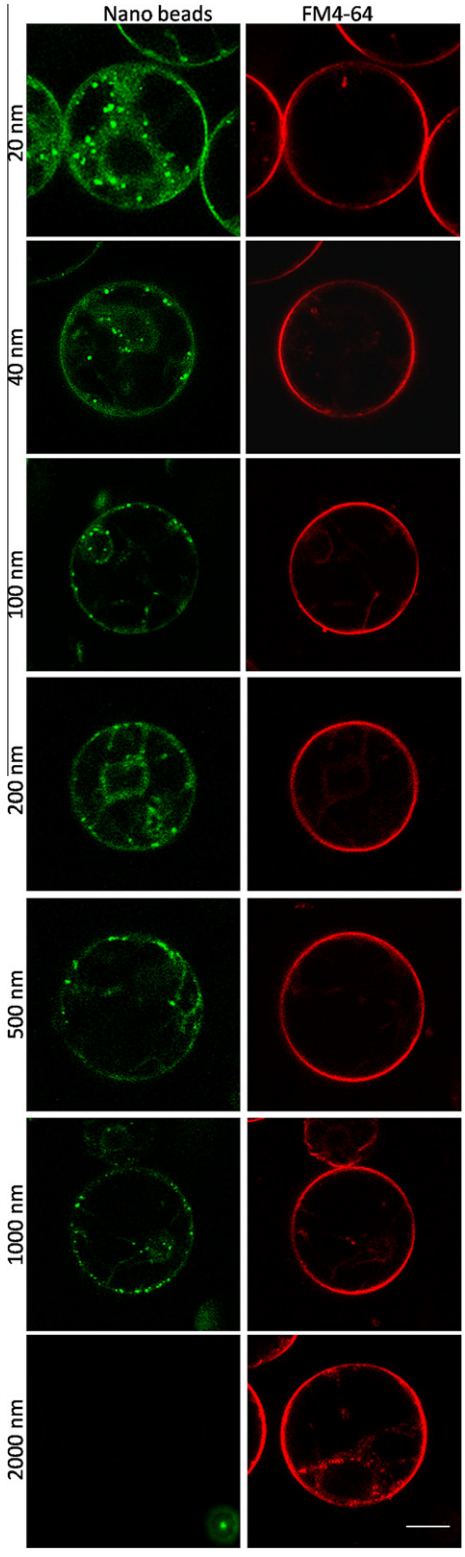


**Fig. 3.** BY-2 protoplasts internalise larger nano beads of up to 1000 nm. Fluorescent images of BY-2 protoplasts incubated with nano beads of different sizes (as indicated). Nano beads from 20 to 1000 nm in diameter are located in bright spots in the cytosol. There is no difference in the localization of beads of different size. Only nano beads with a diameter of 2000 nm are not internalised. Scale bar = 10 μm.

nano bead channel (Fig. 1D). The diffuse signal in the cytosol likely corresponds to small vesicles below the resolution limit. As the resolution limit of the confocal microscope is around 250 nm most of the endocytic vesicles cannot be resolved. However, if the

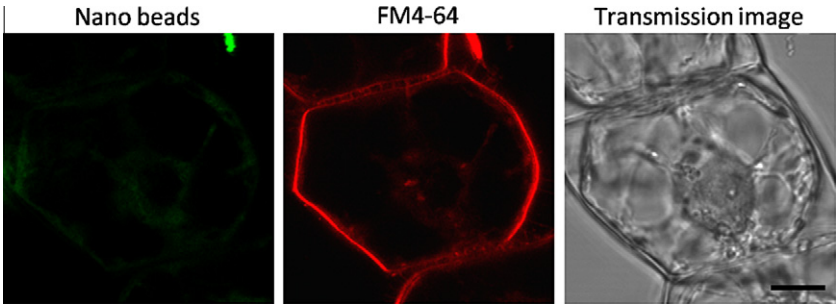


**Fig. 4.** Size limitation of endocytic uptake into BY-2 cells does not depend on turgor pressure. Fluorescent image of plasmolysed BY-2 cells incubated with 100 nm beads. Nano beads are not internalised even if turgor pressure is reduced. The intracellular FM4-64 fluorescence indicates that endocytosis occurred normally these cells. Scale bar = 10 μm.

fluorescent intensity of their cargo is sufficiently high these vesicles can well be detected by fluorescent microscopy. The signal arising from these vesicles leads to a diffuse appearance in the cytosol.

Together the results demonstrate that the 20 nm nano beads are indeed internalised via endocytosis and use at least partially the same endocytic pathway as FM4-64. The results thus demonstrate that fluorescent nano beads provide a useful marker to follow endocytosis in plants.

### *3.2. Different size limitation for endocytic uptake of fluorescent nano beads in turgescent BY-2 cells and protoplasts*

In principle, uptake of particles via fluid-phase endocytosis should be restricted by the size of the endocytic vesicles involved in this process. A size limit for the endocytic uptake of particles would thus provide information on the size of the vesicles involved. We therefore analysed the uptake of larger nano beads into

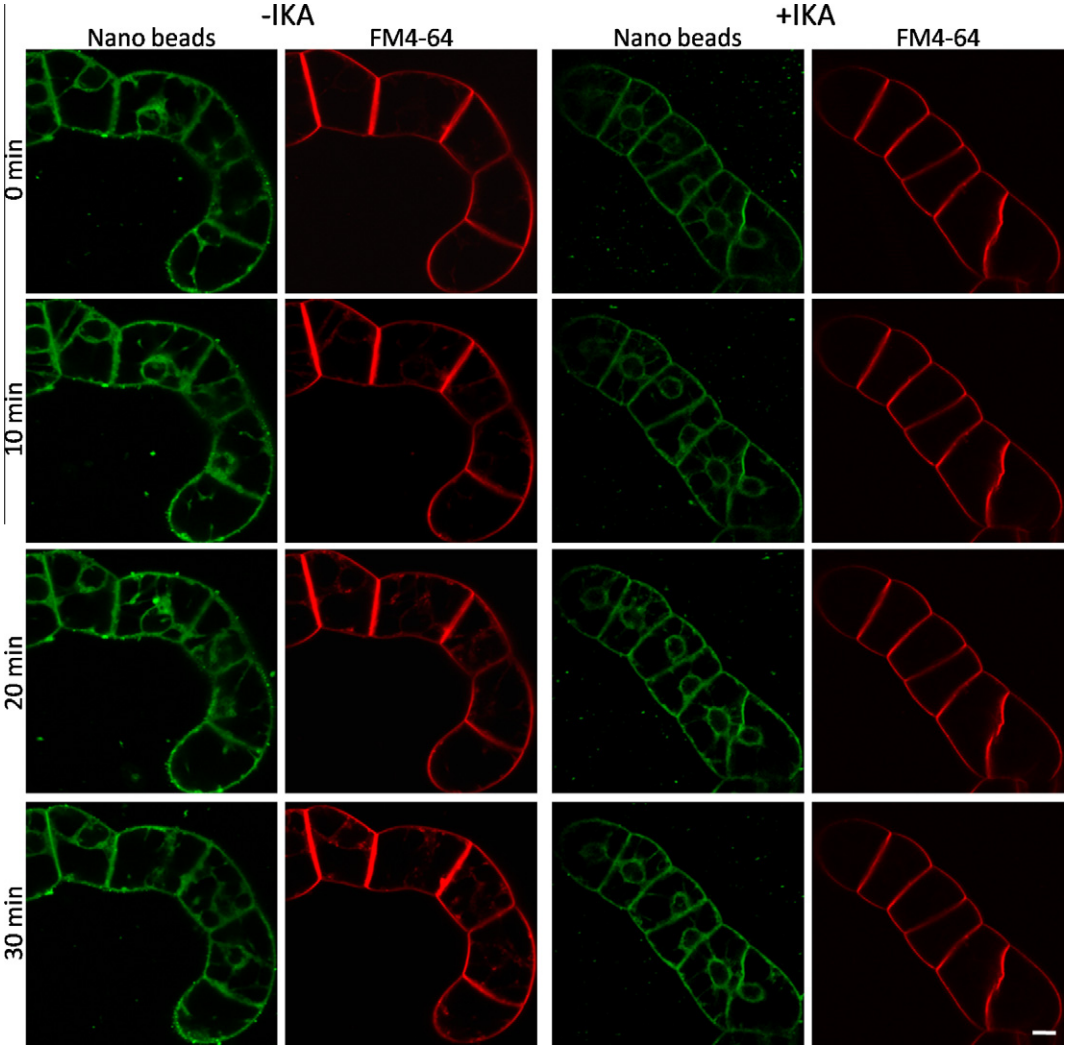


**Fig. 5.** Inhibition of clathrin-mediated endocytosis by IKA does affect uptake of FM4-64 and nano beads. Time series of bead internalisation is shown for untreated cells and cells which were incubated 30 min in 10 μM IKA (as indicated above the panels). Time 0 min corresponds to start of observation, i.e. 2–5 min after addition of nano beads and FM4-64. In control cells intracellular green fluorescence indicates uptake of nano beads. FM4-64 was also internalised. In IKA treated cells almost none FM4-64 uptake was visible demonstrating the block of clathrin-mediated endocytosis by the drug. Internalisation of nano beads was less affected by IKA. Scale bar = 10 μm.

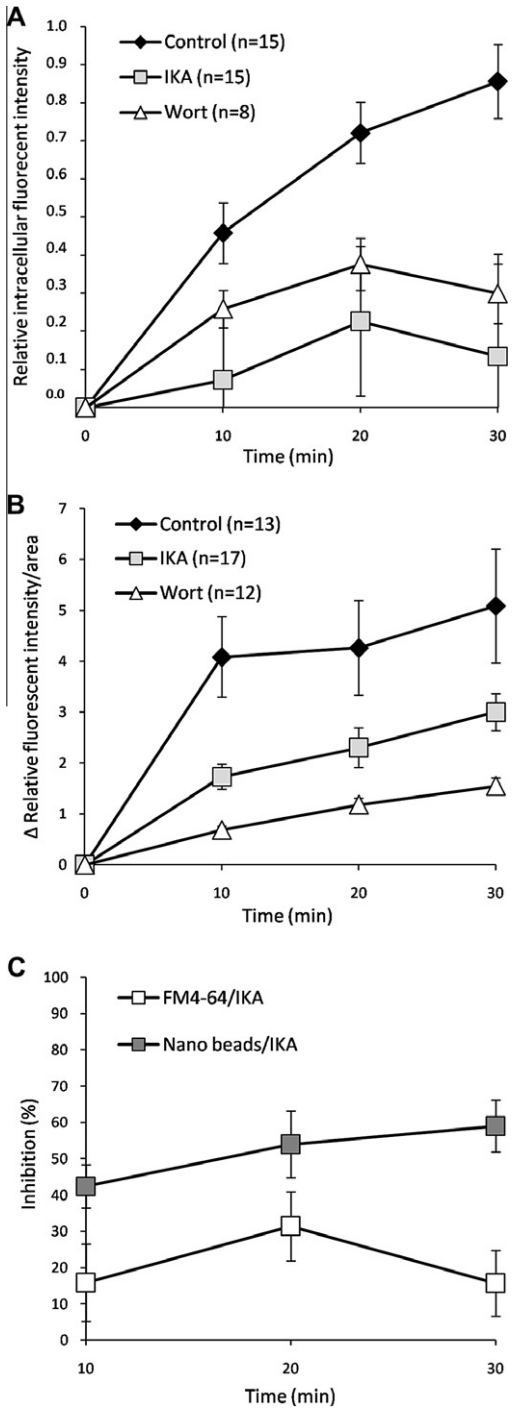


**Fig. 6.** Quantification of the inhibitory effect IKA and wortmannin on the uptake of FM4-64 and nano beads. (A) Uptake of FM4-64. The relative fluorescent intensity is given as the ratio of intracellular fluorescence to whole-cell fluorescence. In control cell the fast increase over time shows the uptake of FM4-64. In IKA treated cell the fluorescent ratio changed barely over time. In wortmannin treated cells the increase in the ratio is strongly reduced. The reduction demonstrates the inhibition of FM4-64 uptake by IKA and wortmannin. (B) Uptake of nano beads. For quantification the intracellular fluorescent intensity per area was estimated. Values are given as the change in fluorescence relative to the start of observation. The increase in control cells represents the continuous uptake of beads over time. IKA and wortmannin reduced endocytosis but did not completely block uptake of nano beads. The block by wortmannin on the uptake of nano beads was significantly larger than the block by IKA (Student's *t*-test, $p = 0.06$). (C) Comparison of the inhibitory effect of IKA on uptake of FM4-64 and nano beads. Inhibition is given as the difference between mean uptake in control cells (set to 100%) and cells treated with IKA. The inhibitory effect of IKA on the uptake of FM4-64 and nano beads was significantly different according to Student's *t*-test ($p = 0.01$). Error bars represent ±SEM.

BY-2 cells. Fig. 2 demonstrates that beads with a diameter of 40 nm are still internalised rapidly. The intracellular fluorescent staining was not different from cells incubated in 20 nm beads. When adding larger nano beads with a diameter of 100 nm no intracellular fluorescence could be detected (Fig. 2). In these cells uptake of FM4-64 occurred normally demonstrating that endocytosis was not inhibited (Fig. 2). The exclusion of nano beads above 100 nm may suggest that endocytic vesicles are too small to allow internalisation of these particles. This would be in agreement with electron microscopy studies which imply that the diameter of endocytic vesicles is typically in the range of 70–180 nm [9]. However, membrane capacitance measurements on BY-2 protoplasts suggested that cells are able to form larger endocytic vesicles. In membrane capacitance studies of constitutive and glucose stimulated endocytosis vesicles of up to 500 nm in diameter were commonly observed [4,10]. In some rare cases endocytic events of vesicles with 900 nm have been recorded [10].

We therefore analysed the uptake of nano beads into BY-2 protoplasts. Fig. 3 shows representative examples of protoplasts 10–20 min after addition of fluorescent nano beads and FM4-64. Internalisation of beads with a diameter of 20 and 40 nm was similar to what has been observed in walled BY-2 cells (Fig. 3, first two rows). However, in contrast to walled cells, BY-2 protoplasts were able to internalise also larger nano beads with a diameter of up to 1000 nm (Fig. 2). There was no detectable difference in the uptake and intracellular distribution of beads of different sizes. Only beads with a diameter of 2000 nm were excluded from internalisation (Fig. 2, last row). This suggests that protoplasts are able to form endocytic vesicles of at least 1000 nm which is much larger than the size of endocytic vesicles typically found in electron microscopy studies of turgescent cells.

This difference could in principle be explained by a lack of turgor in protoplasts. The turgor of walled BY-2 cells may prevent formation of larger vesicles. To test this hypothesis we investigated the uptake of 100 nm nano beads into plasmolysed BY-2 cells. As demonstrated in Fig. 4 plasmolysed cells did also not internalise these beads. Uptake of FM4-64 occurred normally in these cells demonstrating that endocytosis was not inhibited (Fig. 4). The fact that plasmolysed cells do not internalise larger beads suggests that turgor is not the limiting factor for the uptake of larger nano beads. It seems likely that larger beads cannot pass the cell wall due to restricted diffusion through the cell wall pores. This in turn implies that it is not possible to decide from the uptake of fluorescent beads alone if turgescent cells are in principle able to form larger vesicles.

Considering that there are some reports on bigger endocytic vesicles in plants (see for example [1]) we hypothesise that turgescent cells are able to form endocytic vesicles with a diameter larger than the generally reported 70–180 nm. As these vesicles are only rarely formed and may not involve clathrin-coat formation they can hardly be identified in electron microscopy studies. Formation of larger endocytic vesicles may for example account for the bulk uptake of external nutrients such as glucose via fluid-phase endocytosis.

### 3.3. Endocytosis of nano beads involves clathrin-depended and clathrin-independent mechanisms

Studies on the mechanism of endocytosis in plants have so far mainly focused on clathrin-dependent endocytosis. However, there is also evidence for clathrin-independent endocytosis in plants. In particular, endocytic uptake of glucose into BY-2 cells has been shown to occur via a clathrin-independent mechanism [4]. To analyse the role of clathrin in internalisation of nano beads we applied Ikarugamycin (IKA), an inhibitor of clathrin-mediated endocytosis [11,1,2]. Cells were incubated in 40 nm nano beads and FM4-64.

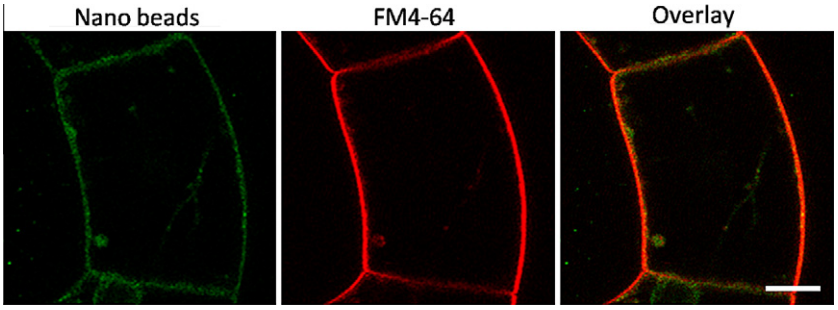


**Fig. 7.** Inhibition of endocytosis by 10 μM wortmannin Fluorescent image of a BY-2 cell treated 30 min with 10 μM wortmannin. Uptake of nano beads was hardly reduced and beads accumulate mainly in wortmannin induced small vacuoles. Scale bar = 10 μm.

Fig. 5 shows a representative example of control cells and cells treated with 10 μM IKA. In control cells staining of the endomembrane system by FM4-64 was already visible 10 min after start of the observation (2–5 min after addition of FM4-64) (Fig. 5). In contrast, IKA treated cells showed almost no FM4-64 uptake (Fig. 5). This is in agreement with previous investigations on Arabidopsis root cells which demonstrated that internalisation of FM4-64 is mainly mediated by clathrin-dependent endocytosis [6]. For quantification of endocytosis of FM4-64 in BY-2 cells we estimated the ratio of intracellular (membrane) fluorescence versus whole-cell fluorescence. Fig. 6A shows a fast increase in relative fluorescence over time (Fig. 6A). In BY-2 cells incubated in IKA the relative fluorescence barely changed reaching a maximum of 30% of control cells (Fig. 6C). This demonstrates that endocytosis of FM4-64 is strongly inhibited by IKA and depends on clathrin-mediated endocytosis. In contrast to the strong block of FM4-64 uptake by IKA, internalisation of fluorescent nano beads was less affected by the drug. In control cells intracellular fluorescence of nano beads was already intense at the beginning of the observation (2–5 min after addition of nano beads; Fig. 5, first row) and hardly changed over the following 30 min (Fig. 5, last row). In IKA treated cells intracellular fluorescence was clearly reduced and slightly increased over time (Fig. 5). Quantification of uptake of nano beads reveals that the relative fluorescent intensity per area was reduced to about half in IKA treated cells after 10 min of observation (Fig. 6B). After 30 min the intracellular fluorescence reached only 60% of control cells implying that clathrin-dependent endocytosis is involved in the uptake of nano beads into BY-2 cells (Fig. 6C). However, the inhibitory effect of IKA on endocytosis of nano beads was significantly less pronounced compared to the reduction of FM4-64 internalisation by IKA (Fig. 6C). This suggests that a second endocytic mechanism which is independent of clathrin coat formation plays a crucial role in the internalisation of nano beads.

To get further insight into the mechanisms involved in endocytosis of nano beads we analysed the effect of wortmannin, a specific inhibitor of the phosphatidylinositol (PI) 3-kinases [12]. Studies by Lam et al. [13] suggest that wortmannin acts downstream of the early endosome by inhibiting the further transport of vesicles resulting in accumulation of cargo in small vacuoles also known as 'wortmannin compartments'. Recent studies by Ito et al., [14] imply that the inhibitory effect of wortmannin on endocytosis is also due to the aggregation and stabilization of clathrin-coated pits at the plasma membrane.

In BY-2 cells the treatment with 10 μM wortmannin for 30 min led to a severe inhibition of FM4-64 uptake and to an accumulation of the dye in small vacuoles (Fig. 7). Quantification of the inhibitory effect of wortmannin revealed a strong block of FM4-64 uptake similar to the one observed in IKA treated cells (Fig. 6A). The pronounced reduction of uptake of FM-64 by wortmannin is in agreement with previous studies on endocytosis in plant cells [13] and is most likely due to the inhibitory effect of wortmannin on clathrin-mediated endocytosis [14].

In wortmannin treated cells the internalisation of nano beads was also strongly reduced (Fig. 7) demonstrating that endocytosis of nano beads depends on the function of PI3-kinases. Internalised beads mainly accumulated in the 'wortmannin compartments' also labelled by FM4-64. This suggests that the endocytic pathway of nano beads also includes endosomes as intermediate compartments.

Quantification of the inhibitory effect of wortmannin on the uptake of nano beads shows that the block of endocytosis of nano beads by wortmannin was significantly larger than the inhibition by IKA (Fig. 6B, Student's $t$-test, $p = 0.06$). This demonstrated that he uptake of nano beads involves an additional wortmannin-dependent but clathrin-independent endocytic process. In mammalian cells wortmannin is known as a competent inhibitor of macropinocytosis [15]. Macropinocytosis is preliminary used for the non-selective internalisation of fluid and membrane and is exploited by pathogens for cellular invasion [16]. The process was also found to be responsible for the uptake of silver nanoparticles (diameter 80 nm) in human cells [17]. One may therefore speculate that clathrin-independent endocytosis of nano beads in BY-2 cells shares some mechanisms with macropinocytosis in mammalian cells. Future research will show if macropinocytosis is indeed an evolutionary conserved endocytic pathway with similar mechanisms in mammalian and plant cells.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.08.008.

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